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**U.S. PATENT APPLICATION**

Title: **CEREBRAL ORGANIC ANION TRANSPORTER AND ITS GENE**

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# CEREBRAL ORGANIC ANION TRANSPORTER AND ITS GENE

## Technical Field

The present invention relates to a gene involved in organic negative ion (organic anion) transport and the polypeptide encoded by the gene.

## Background Art

Liver and kidney play important roles in the metabolism and excretion of biologically foreign compounds and drugs out of bodies. Tubule cells and hepatocytes belong to epithelial cells with polarities. It is supposed that some of anionic substances are taken up through the basolateral membranes into kidney and liver by transporters, while the organic anions generated metabolically in cells are excreted by transporters.

The uptake of organic anions through the basolateral membranes of tubule cells and hepatocytes have been investigated so far in experiment systems using isolated organ perfusion protocols, dissected cells and membrane vesicles. According to such conventional methods, however, the detailed analysis of the transport of organic anions through the basolateral membranes has been difficult. Accordingly, it has been desired to isolate the transporters per se and analyze the properties of transporters in detail.

Alternatively, plural experimental results suggestively indicate the presence of the transport of organic anions in brain. The transport of organic anions in brain is supposed to function for the extracerebral excretion of endogenous and exogenous organic anions.

Although the transport of organic anions in brain is speculated to play an

important role in the elimination of endogenous anions and foreign compounds from brain, the detail of the transport therein is more ambiguous than the transport in kidney and liver, due to the difficulty in physiological experiments therein.

Based on these backgrounds, the screening of the organic anion transporter molecules per se has been actively carried out in 1990 and thereafter. Consequently, two organic anion transporters derived from the basolateral membrane of liver have been isolated until the last year. (Hagenbuch, B. et al., Proc. Natl. Acad. Sci. USA, Vol. 88, pp. 10629-33, 1991; Jacquemin, E. et al., Proc. Natl. Acad. Sci. USA, Vol. 91, pp. 133-7, 1994)

The present inventors independently isolated an organic anion transporter OAT1 responsible for the most important function in the organic anion transport in kidney successfully last year (Sekine, T., et al., J. Biol. Chem., Vol. 272, pp. 18526-9, 1997) and already filed the patent application thereof. OAT1 is a transporter capable of transporting a great number of organic anions with different chemical structures and is also involved in the transport of various anionic drugs. OAT1 is expressed in a specific manner to kidney, while OAT1 is very slightly expressed in brain except kidney.

Recently, the inventors have further identified a liver-specific organic anion transporter (OAT2) with about 40 % homology to OAT1 in terms of amino acid level (FEBS letter, Vol. 429, pp. 179-182, 1998) (Japanese Patent Application No. 169174/1998).

The isolation and identification of OAT1 and OAT2 indicates that these organic anion transporters form a family. Additionally because OAT2 is expressed specifically in liver, it is suggested that the family is not kidney-specific but is expressed in various organs.

As described insofar, it is suggested that an organic anion transport system is present in brain, but the OAT1 expression in brain is quite slight while OAT2 is not present therein. Based on these findings, the inventors have anticipated the presence of an unknown transporter responsible for the organic anion transport in brain.

Alternatively, the organic anion transport in the basolateral membrane of liver is complicated; particularly, the efflux flow of conjugated substances (many of the conjugated substances are organic anions) generated at a vast scale in hepatocytes into blood has not yet been known. The organic anion transport in liver cannot sufficiently be described on the single basis of the organic anion transporters including OAT2. Hence, the presence of an unknown transporter is suggested.

The inventors isolated the organic anion transporter OAT1 serving as the most important role in the organic anion transport in kidney (Sekine, T. et al., J. Biol. Chem. Vol. 272, pp. 18526-9, 1997). Based on the structural similarity to OAT1, the inventors identified a liver-specific organic anion transporter (OAT2) (Sekine, T., et al., FEBS letter, Vol. 429, pp. 179-182, 1998). The inventors already reported additionally (Sekine, T., et al., J. Biol. Chem., Vol. 272, pp. 18526-9, 1997) that OAT1 had low homology to an organic cation transporter OCT1 (Grundemann, D. et al., Nature, Vol. 372, pp. 549-52, 1994).

Taking account of these evidence, the inventors identified a sequence common to OAT1, OAT2 and OCT1 and prepared a degenerate primer based on the sequence. By using the degenerate primer, the inventors identified a novel cDNA fragment with low homology to OAT1, OAT2 and OCT1 from rat brain mRNA by RT (reverse transcript)-PCR (polymerase chain reaction) method. By using the cDNA fragment, a cDNA never reported yet was discriminated from the rat cDNA library.

The resulting protein was designated cerebral type organic anion transporter OAT3 as a third member of the OAT family.

Disclosure of the Invention

The invention relates to the organic anion transporter OAT3. The inventive organic anion transporter OAT3 is a transporter with a wide range of substrate selectivity and transports organic anions with different chemical structures (having a potency to take up the organic anions). However, no substantial uptake of a typical organic cation TEA (tetraethylammonium) is observed. Hence, the inventive organic anion transporter OAT3 with a wide range of substrate selectivity is an organic anion transporter with no substantial substrate selectivity of TEA (tetraethylammonium) as the typical organic cation but is selectively distributed in organs mainly including brain and liver.

The inventive protein includes the organic anion transporter OAT3 of an amino acid sequence represented by SQ ID No. 2 (in human) or 4 (in rat) or of an amino acid sequence with such a modification of the aforementioned amino acid sequence as deletion, substitution or addition of one or several amino acids. The deletion, substitution or addition of amino acids is satisfactory at an extent such that no organic anion transport activity is deteriorated; the number of the amino acids then is generally 1 to about 110, preferably 1 to about 55. Such protein has generally 60 to 80 %, preferably 70 to 90 % homology in amino acid sequence to the amino acid sequence represented by SQ ID No. 2 or 4.

Furthermore, the invention encompasses a nucleic acid, preferably DNA or RNA, encoding the inventive protein comprising the organic anion transporter OAT3. The inventive nucleic acid encompasses the nucleic acid encoding the inventive

protein and nucleic acids hybridizable with the nucleic acid under stringent conditions.

Still furthermore, the invention relates to a partial sequence of the nucleic acid encoding the inventive protein or nucleotides hybridizable with the partial sequence under stringent conditions.

Still yet furthermore, the invention relates to an antibody against the inventive protein or a polypeptide immunologically identical to the inventive protein.

Brief Description of the Drawings

Fig. 1 depicts the organic anion uptake activity of the inventive rat OAT3 expressed in Xenopus oocyte;

Fig. 2 depicts the results of kinetic analyses of the transport of PAH, estrone sulfate and ochratoxin A with the inventive rat OAT3 in the oocyte;

Fig. 3 depicts the results on the inhibition of the organic anion transport with the inventive rat OAT3 by various organic substances;

Fig. 4 depicts the results of the Northern blotting analysis of the inventive rat OAT3 gene;

Fig. 5 depicts the results on the inhibition of the rat OAT3 transport by various metabolites of cerebral type neurotransmitters;

Fig. 6 depicts the uptake activity of $^{14}$C-PAH (p-aminohippuric acid) when the inventive hOAT3 was expressed in Xenopus oocyte;

Fig. 7 depicts the uptake activity of $^{3}$H-estrone sulfate when the inventive hOAT3 was expressed in Xenopus oocyte;

Fig. 8 depicts the uptake activity of $^{3}$H-dehydroepiandrosterone sulfate when the inventive hOAT3 was expressed in Xenopus oocyte;

Fig. 9 depicts the uptake activity of $^{3}$H-ochratoxin A when the inventive hOAT3 was expressed in Xenopus oocyte;

Fig. 10 depicts the uptake activity of $^{3}$H-cimetidine when the inventive hOAT3 was expressed in Xenopus oocyte;

Fig. 11 depicts the uptake activity of $^{3}$H-estradiol glucuronide when the inventive hOAT3 was expressed in Xenopus oocyte;

Fig. 12 depicts the uptake activity of $^{3}$H-prostaglandin E2 when the inventive hOAT3 was expressed in Xenopus oocyte;

Fig. 13 depicts the uptake activity of $^{14}$C-taurocholic acid when the inventive hOAT3 was expressed in Xenopus oocyte;

Fig. 14 depicts the uptake activity of $^{14}$C-glutaric acid when the inventive hOAT3 was expressed in Xenopus oocyte;

Fig. 15 depicts the uptake activity of $^{3}$H-methotrexate when the inventive hOAT3 was expressed in Xenopus oocyte;

Fig. 16 depicts the uptake activity of $^{14}$C-salicylic acid when the inventive hOAT3 was expressed in Xenopus oocyte;

Fig. 17 depicts the uptake activity of $^{14}$C-indomethacin when the inventive hOAT3 was expressed in Xenopus oocyte;

Fig. 18 depicts the uptake activity of $^{14}$C-cholic acid when the inventive hOAT3 was expressed in Xenopus oocyte; and

Fig. 19 depicts the results on the inhibition of the transport of $^{3}$H-estrone sulfate with the inventive hOAT3 by various organic substances.

## Best Mode for Carrying out the Invention

The inventive organic anion transporter gene can be isolated and identified

by screening of tissues and cells of organs such as kidney and brain in appropriate mammalian animals used as gene sources. The mammalian animals include non-human animals such as dog, cow, horse, goat, sheep, monkey, pig, rabbit, rat and mouse and additionally include human.

The gene screening and isolation can preferably be carried out by homology screening and PCR screening. The base sequence of the resulting cDNA is determined by a conventional method; the translation region is analyzed; and the amino acid sequence of the protein encoded by the cDNA, namely the amino acid sequence of OAT3, can be determined.

It is verified for example by the following manners that the resulting cDNA is the cDNA of the organic anion transporter gene, namely that the genetic product encoded by the cDNA is the organic anion transporter. More specifically, the cRNA prepared from the isolated OAT3 gene is integrated and expressed in the oocyte; then, the transport (uptake) potency of organic anions in cells is confirmed by assaying the incorporation of an appropriate organic anion as the substrate in cells by the general uptake experiment (Sekine, T., et al., J. Biol. Chem., Vol. 272, pp. 18526-9, 1997).

By applying the same uptake experiment to the expression cell, the transport property and substrate specificity of OAT3 can be examined.

The SQ ID No. 3 in the sequence listing shows the base sequence of the cDNA of the rat organic anion transporter OAT3 isolated by such method; and SQ ID No. 4 shows the amino acid sequence thereof.

By using the cDNA of the resulting OAT3 gene for screening an appropriate cDNA library or genomic DNA library prepared by using a different gene source, a homologous gene or chromosomal gene derived from a different tissue or a different biological organism or the homology can be isolated.

The base sequence of the cDNA of human organic anion transporter OAT3 identified by such method is shown as SQ ID No. 1 and the amino acid sequence thereof is shown as SQ ID No. 2.

By using a synthetic primer designed on the basis of the base sequence as the base sequence (SQ ID No. 1 or 3) of the inventive gene disclosed or a part of the information thereof, the gene can be isolated from the cDNA library by general PCR.

DNA libraries such as cDNA library or genomic DNA library or the like can be prepared by the method described in for example "Molecular Cloning; Sambrook, J., Fritsh, E. F. and Maniatis, T. ed., issued by Cold Spring Harbor Laboratory Press in 1989". Otherwise, any existing commercially available library can satisfactorily be used.

The inventive organic anion transporter (OAT3) can be generated by using for example cDNA encoding the organic anion transporter by genetic recombinant technology. For example, DNA (cDNA and the like) encoding the organic anion transporter is integrated in an appropriate expression vector; and the resulting recombinant DNA can then be transfected in an appropriate host cell. The expression system (host vector system) for polypeptide generation includes for example expression systems of bacteria, yeast, insect cells and mammalian cells. Among them, insect cells and mammalian cells are preferably used for the recovery of the functional protein.

For the expression of the polypeptide in mammals, for example, the DNA encoding the inventive organic anion transporter is inserted in the downstream of an appropriate promoter (for example, SV40 promoter, LTR promoter, elongation 1α promoter and the like) in an appropriate expression vector (for example, retrovirus vector, papilloma virus vector, vaccinia virus vector, SV40 vector and the like) to

construct an expression vector. By subsequently transforming an appropriate animal cell with the resulting expression vector and culturing the transformant in an appropriate culture medium, the objective polypeptide can be generated. The mammalian cell as the host includes monkey COS-7 cell, Chinese hamster CHO cell, human HeLa cell, or cell lines such as kidney tissue-derived primary culture cell, porcine kidney-derived LLC-PK1 cell and opposum kidney-derived OK cell and the like.

As the cDNA encoding the organic anion transporter OAT3, use can be made of cDNA with the base sequence represented by SQ ID No. 1 or 3; as the cDNA, with no specific limitation to the cDNA described above, additionally, DNA corresponding to the amino acid sequence is designed and used, which can encode the polypeptide. In this case, it is known that each amino acid is encoded by one to 6 types of codons, so codons for use can be selected appropriately. For example, a sequence with higher expression can be designed, in terms of the frequency of codons used by a host for expression. DNA with the designed base sequence can be recovered by chemical DNA synthesis, fragmentation and conjugation of the cDNA, and a partial modification of the base sequence. An artificial partial modification of the base sequence or mutagenesis thereof can be carried out by site specific mutagenesis, by utilizing a primer comprising a synthetic oligonucleotide encoding the desired modification "Mark, D. F., et al., Proc. Natl. Acad. Sci. USA, Vol. 8, pp. 5662-5666, 1984".

Nucleotides (oligonucleotide or polynucleotide) hybridizable with the inventive organic anion transporter gene under stringent conditions can be used as probe for detecting the organic anion transporter gene and can also be used for example as antisense oligonucleotide, ribozyme and decoy, so as to modify the

expression of the organic anion transporter.

In accordance with the invention, the term hybridization under stringent conditions generally means hybridization in 5 × SSC or a hybridization solution at a salt concentration equal to the concentration under a temperature condition of 37 to 42 °C for about 12 hours, followed by preliminary rinsing in 5 × SSC or a solution at a salt concentration equal to the concentration and rinsing in 1 × SSC or at a salt concentration equal to the concentration. Higher stringency can be realized by carrying out rinsing in 0.1 × SSC or a solution at a salt concentration equal to the concentration.

Additionally, the invention relates to a partial sequence of the nucleic acid encoding the inventive protein or nucleotides hybridizable with the sequence under stringent conditions. As such nucleotides, generally, use can satisfactorily be made of nucleotides comprising a partial sequence of consecutive 14 or more nucleotides in series in the base sequence represented by SQ ID No. 1 or 3 or a sequence complementary to the partial sequence; so as to enhance the specificity of the hybridization, a longer sequence, for example a sequence of 20 bases or more or a sequence of 30 bases or more, can satisfactorily be used as such partial sequence. These nucleotides can be labeled, if necessary, with radioactive elements, fluorescent substances or chemiluminescent substances.

The nucleotides comprising a partial sequence of consecutive 14 or more base in series in the inventive base sequence represented by SQ ID No. 1 or 3 or a sequence complementary to the partial sequence preferably carries the specific base sequence of the base sequence encoding the inventive organic anion transporter OAT3 and can satisfactorily be labeled, if necessary.

By using the inventive organic anion transporter or a polypeptide

immunologically identical thereto, additionally, an antibody can be raised. The antibody can be utilized for detecting or purifying the organic anion transporter. The antibody can be raised, by using the inventive organic anion transporter, a fragment thereof, or a synthetic peptide with a partial sequence thereof or the like as an antigen. The antibody, if polyclonal, can be generated by general methods comprising inoculating such antigen in a host animal (for example, rat and rabbit) and recovering the resulting immunized serum. The antibody, if monoclonal, can be generated by techniques such as general hybridoma method. Further, the inventive antibody is satisfactorily prepared as chimera form or humanized antibody.

Best Mode for Carrying out the Invention

The description is now made in more detail in the following examples, but the examples are in no way of limitation of the invention.

In the following examples, the individual procedures followed the methods described in "Molecular Cloning; Sambrook, J., Fritsh, E. F. and Maniatis, T. ed., issued by Cold Spring Harbor Laboratory Press in 1989" or followed the instructions of commercially available kit products if used, unless otherwise stated.

Example 1

Isolation and analysis of multi-selective organic anion transporter 3 (OAT3) cDNA

(1) Preparation of degenerate primer based on the base sequence information of OAT1, OAT2 and OCT1

Based on the base sequence information of OAT1 and OAT2 isolated previously by the inventors and the reported base sequence information of OCT1, degenerate primer was prepared with reference to amino acid sequences in common to

these three transporters (amino acids 267-275 and amino acids 447-452 in the amino acid sequence of OAT1).

From rat brain was extracted total RNA by GITC method; and poly(A) + RNA was then purified by using an oligodT column. From the rat brain poly(A) + RNA was prepared cDNA by using reverse transcriptase; using the resulting cDNA as template, PCR was conducted with the degenerate primer. Consequently, a PCR product of about 550 bp was prepared.

By using a TA cloning kit (manufactured by Invitrogen Co.), the PCR product was cloned; and some of the base sequence was determined. Consequently, a novel cDNA (B10) with homology at the level of 50 % to OAT1 in terms of amino acid level was recovered.

A probe prepared by labeling B10 cDNA with $^{32}P$ was used for Northern hybridization with poly(A) + RNA extracted from various rat organs. Positive bands were visually detected in the liver, kidney, brain and eyes.

Because the inventors had an excellent cDNA library of rat kidney, the inventors screened the rat kidney cDNA library by using the B10 probe. Hybridization was promoted overnight in a hybridization solution at 37 °C. Thereafter, the filter membrane was rinsed in 0.1 × SSC/0.1 % SDS at 37 °C. As the hybridization solution, use was made of a buffer, pH 6.5 containing 50 % formamide, 5 × standard saline citrate (SSC), 3 × Denhard solution, 0.2 % SDS, 10 % dextran sulfate, 0.2 mg/ml modified salmon sperm DNA, 2.5 mM sodium pyrophosphate, 25 mM MES, and 0.01 % Antifoam B (manufactured by Sigma, Co.). The clone isolated in λZipLox was further subcloned in a plasmid vector pZL by in vivo excision method. Consequently, a novel clone (rk1411) with an organic anion transport activity was recovered (Example 2 below is to be referenced concerning transport function

analysis).

The base sequence of the clone (rk1411) recovered above was determined as follows. By firstly using a kilo-sequence deletion kit (manufactured by TaKaRa, Co.), plural plasmid DNAs were prepared by subjecting the clone rk1411 to each deletion of about 300 bp from the single side thereof. The base sequences of the DNAs were determined by using an automatic sequencer (manufactured by Applied BioSystems). Additionally, a specific oligonucleotide primer for rk1411 was prepared; by using the automatic sequencer, the base sequences thereof were also analyzed from the opposite direction. Finally, the whole base sequence of rk1411 was determined. The base sequence is shown as SQ ID No. 3 in the sequence listing. Additionally, the amino acid sequence of the protein is shown as SQ ID No. 4.

Example 2 (Identification of the function of rk1411)

(1) By using T7 RNA polymerase, cRNA (RNA complementary to cDNA) was prepared in vitro from the plasmid carrying the clone (rk1411) as described above (see Sekine, T., et al. J. Biol. Chem., Vol. 272, pp. 18526-9, 1997).

According to the method already reported (Sekine, T., et al. J. Biol. Chem., Vol. 272, pp. 18526-9, 1997), the resulting cRNA was injected in the Xenopus oocyte; the oocyte was subjected to an uptake test with various radiolabeled organic anions and organic cations. As shown in Fig. 1, consequently, the oocyte in which rk1411 was expressed could take up $^{14}$C-PAH (p-aminohippuric acid), $^{3}$H-ochratoxin A and $^{3}$H-estrone sulfate. Alternatively, the oocyte never transported one typical organic cation $^{14}$C-TEA (tetraethylammonium).

The organic anion transport with rk1411 was subjected to the Michaelis-Menten dynamic test. By examining the change in the uptake of PAH, estrone

sulfate and ochratoxin A at various concentrations, the dependency of the rk1411 transport on the concentrations of these substrates was examined. The uptake experiments of radiolabeled PAH, estrone sulfate and ochratoxin A were carried out by using the oocyte injected with rk1411 cRNA according to the method described above. The results are as follows (see Fig. 2): the Km values of PAH, estrone sulfate and ochratoxin A were 4.7 μM, 2.3 μM and 0.74 μM, respectively. The results are shown below in Table 1.

Table 1

Results of Michaelis-Menten dynamic test

| | Km (μM) | Vmax (pmol/hr/oocyte) | Vmax/Km (μl/hr/oocyte) |
|---|---|---|---|
| PAH | 64.7 ± 10.0 | 23.3 ± 2.8 | 0.360 |
| Estrone sulfate | 2.34 ± 0.20 | 7.60 ± 0.44 | 3.24 |
| Ochratoxin A | 0.739 ± 0.178 | 3.08 ± 0.33 | 4.17 |

(2) So as to examine the substrate selectivity of rk1411, various anionic substances were added to the $^{3}$H-estrone sulfate uptake experiment system with the oocyte injected with rk1411 cRNA, to examine their influences (inhibition experiment). The $^{3}$H-estrone sulfate uptake experiment was conducted by using the oocyte injected with rk1411 cRNA according to the method described above. In the presence and absence of 1 mM each compound (with no label), the uptake of $^{3}$H-estrone sulfate was assayed. Consequently, various anionic substances (taurocholic acid, cholic acid, bromosulfophthalein, probenecid, indocyanine green, bumetanide, cefoperazone, pyroxicam, furosemide, azidothymidine, benzylpenicillin and the like) significantly inhibited the $^{3}$H-estrone sulfate transport with rk1411 (see Fig. 3). Meanwhile, cationic substances such as tetraethylammonium, guanidine, quinidine and berapamil never exerted any such inhibitory action (see Fig. 3). The results indicate

that rk1411 is a multi-selective transporter and primarily recognizes organic anions. Hence, rk1411 was designated OAT3 (organic anionic transporter 3) as a third member of the OAT family.

Example 3

The expression of the OAT3 gene in individual rat tissues was analyzed (Northern blotting). The OAT3 cDNA in the whole length was labeled with $^{32}$P-dCTP; by using the resulting cDNA as probe, RNAs extracted from various rat tissues were subjected to Northern blotting as follows. 3 μg of poly(A) + RNA was electrophoresed on 1 % agarose/formaldehyde gel and subsequently transferred on a nitrocellulose filter. The filter was hybridized overnight in a hybridization solution containing the whole length of the $^{32}$p-dCTP-labeled OAT3 cDNA at 42 °C. The filter was rinsed in 0.1 × SSC containing 0.1 % SDS at 65 °C.

The Northern blotting results (see Fig. 4) indicate that a strong band was detected around 2.4 Kb in the RNAs from the kidney, liver and brain. Visually weak expression was also observed in the eyes.

Example 4

Because OAT3 was most strongly expressed in brain among the members of the OAT family, an attempt was made to deduce the role thereof in brain at an inhibition experiment of the OAT3 transport with various metabolites of neurotransmitters (mainly organic anions). As shown in Fig. 5, noradrenalin and serotonin metabolites inhibited the OAT3 transport of estrone sulfate, suggesting a possibility that these metabolites per se might be substrates of OAT3. The evidence indicates that OAT3 has an action to excrete neurotransmitter metabolites out of

brain as one function of cerebral type OAT3.

Example 5

Isolation and analysis of human-type multi-selective organic anion transporter 3 (OAT3) cDNA

EST (expressed sequence tag) data base was screened by using the rat OAT3 cDNA isolated previously by the inventors. Human EST clone (H20345) with high homology to the rat OAT3 was identified. A part (333 bp) of the base sequence of the clone was synthesized by PCR. The cDNA fragment was labeled with $^{32}P$, which was then used as probe for the following screening.

The human kidney cDNA library maintained by the inventors was subjected to screening with the probe. Hybridization was effected all day long and overnight in a hybridization solution at 37 °C; subsequently, the filter membrane was rinsed in 0.1 × SSC/0.1 % SDS at 37 °C. As the hybridization solution, use was made of a buffer, pH 6.5, containing 50 % formamide, 5 × SSC (standard saline citrate), 3 × Denhard solution, 0.2 % SDS, 10 % dextran sulfate, 0.2 mg/ml modified salmon sperm DNA, 2.5 mM sodium pyrophosphate, 25 mM MES, and 0.01 % Antifoam B (manufactured by Sigma, Co.). The clone isolated in λZipLox was further subcloned in a plasmid vector pZL by in vitro excision method. Consequently, a novel human organic anion transporter 3 (hOAT3) with an organic anion transport activity was recovered. The analysis of the transport function thereof is described below in Example 6.

The base sequence of hOAT3 was determined by the following method. Oligonucleotide primers specific to hOAT3 were sequentially synthesized. By using an automatic sequencer (manufactured by Applied BioSystems, Co.), the base sequence was analyzed, starting from both the 5'- and 3'-termini. Finally, the whole

base sequence of hOAT3 was determined. The determined base sequence is shown as SQ ID No. 1 in the sequence listing. Based on the cDNA sequence, the amino acid sequence encoding hOAT3 is described as SQ ID No. 2 in the sequence listing.

The base sequence of the cDNA is shown in Table 2, while the amino acid sequence is shown in Table 3, in a corresponding manner.

Table 2

Base sequence of hOAT3 cDNA

```
        10          20          30          40          50          60
CTGAGCTGCC CTACTACAGC AGCTGCCGGC CCCTAGGACA GAGCAGGGAC CTCAACTACA

        70          80          90         100         110         120
CTGATCACCA GCCCCATCGG ATCCAGACCC GGCCACCAGC TCTGGCTCGT CTTGCCCCAG

       130         140         150         160         170         180
TGCCATGACC TTCTCGGAGA TCCTGGACCG TGTGGGAAGC ATGGGCCATT TCCAGTTCCT

       190         200         210         220         230         240
GCATGTAGCC ATACTGGGCC TCCCGATCCT CAACATGGCC AACCACAACC TGCTGCAGAT

       250         260         270         280         290         300
CTTCACAGCC GCCAGCGCGTG TCCACCACTG TCGCCGCGCC CACAATGCCT CCACAGGGCC

       310         320         330         340         350         360
TTGGGTGCTC CCCATGGGCC CAAATGGGAA GCCTGAGAGG TGCCTCCGTT TTGTACATCC

       370         380         390         400         410         420
GCCCAATGCC AGCCTGCCCA ATGACACCCA GAGGGCCATG GAGCCATGCC TGGATGGCTG

       430         440         450         460         470         480
GGTCTACAAC AGCACCAAGG ACTCCATTGT GACAGAGTGG GACTTGGTGT GCAACTCCAA

       490         500         510         520         530         540
CAAACTGAAG GAGATGGCCC AGTCTATCTT CATGGCAGGT ATACTGATTG GAGGGCTCGT

       550         560         570         580         590         600
GCTTGGAGAC CTGTCTGACA GGTTTGGCCG CAGGCCCATC CTGACCTGCA GCTACCTGCT

       610         620         630         640         650         660
GCTGGCAGCC AGCGGCTCCG GTGCAGCCTT CAGCCCCACC TTCCCCATCT ACATGGTCTT

       670         680         690         700         710         720
CCGCTTCCTG TGTGGCTTTG GCATCTCAGG CATTACCCTG AGCACCGTCA TCTTGAATGT

       730         740         750         760         770         780
GGAATGGGTG CCTACCCGGA TGCGGGCCAT CATGTCGACA GCACTCGGGT ACTGCTACAC

       790         800         810         820         830         840
CTTTGGCCAG TTCATTCTGC CCGGCCTGGC CTACGCCATC CCCCAGTGGC GTTGGCTGCA

       850         860         870         880         890         900
GTTAACTGTG TCCATTCCCT TCTTCGTCTT CTTCGTATCA TCCTGGTGGA CACCAGAGTC

       910         920         930         940         950         960
CATACGCTGG TTGGTCTTGT CTGGAAAGTC CTCGGAGGCC CTGAAGATAC TCCGGCGGGT

       970         980         990        1000        1010        1020
GGCTGTCTTC AATGGCAAGA AGGAAGAGGG AGAAAGGCTC AGCTTGGAGG AGCTCAAACT

      1030        1040        1050        1060        1070        1080
CAACCTGCAG AAGGAGATCT CCTTGGCCAA GGCCAAGTAC ACCGCAAGTG ACCTGTTCCG

      1090        1100        1110        1120        1130        1140
GATACCCATG CTGCGCCGCA TGACCTTCTG TCTTTCCCTG GCCTGGTTTG CTACCGGTTT
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      1150       1160       1170       1180       1190       1200
TGCCTACTAT AGTTTGGCTA TGGGTGTGGA AGAATTTGGA GTCAACCTCT ACATCCTCCA

      1210       1220       1230       1240       1250       1260
GATCATCTTT GGTGGGGTCG ATGTCCCAGC CAAGTTCATC ACCATCCTCT CCTTAAGCTA

      1270       1280       1290       1300       1310       1320
CCTGGGCCGG CATACCACTC AGGCCGCTGC CCTGCTCCTG GCAGGAGGGG CCATCTTGGC

      1330       1340       1350       1360       1370       1380
TCTCACCTTT GTGCCCTTGG ACTTGCAGAC CGTGAGGACA GTATTGGCTG TGTTTGGGAA

      1390       1400       1410       1420       1430       1440
GGGATGCCTA TCCAGCTCCT TCAGCTGCCT CTTCCTCTAC ACAAGTGAAT TATACCCCAC

      1450       1460       1470       1480       1490       1500
AGTCATCAGG CAAACAGGTA TGGGCGTAAG TAACCTGTGG ACCCGCGTGG GAAGCATGGT

      1510       1520       1530       1540       1550       1560
GTCCCCGCTG GTGAAAATCA CGGGTGAGGT ACAGCCCTTC ATCCCCAATA TCATCTACGG

      1570       1580       1590       1600       1610       1620
GATCACCGCC CTCCTCGGGG GCAGTGCTGC CCTCTTCCTG CCTGAGACCC TGAATCAGCC

      1630       1640       1650       1660       1670       1680
CTTGCCAGAG ACTATCGAAG ACCTGGAAAA CTGGTCCCTG CGGGCAAAGA AGCCAAAGCA

      1690       1700       1710       1720       1730       1740
GGAGCCAGAG GTGGAAAAGG CCTCCCAGAG GATCCCTCTA CAGCCTCACG GACCAGGCCT

      1750       1760       1770       1780       1790       1800
GGGCTCCAGC TGAGGACAAC GGAACCCCCT TTCCCTGCCC TCCAGAGACT GATCCTAGCC

      1810       1820       1830       1840       1850       1860
AGGCACCTTA GGAGTATAGG GAGGCCCCAT ATAGGTCCAT CCTCCTAGGA TGAAGCCTTC

      1870       1880       1890       1900       1910       1920
TGAGAGCTTG GTGAAGGTGT CTCCATCACC ACCACCAGAG CCTCCTGCCC AGCCCTGGCC

      1930       1940       1950       1960       1970       1980
AGTTCAAAGG TTCAGCCATC CCTGCCCTTG TTCTGCCTGC AACCCAGGCC CTGCCATTCT

      1990       2000       2010       2020       2030       2040
TCTGTCTAGC CCTTCCCCAC TGGCCACCTT CCCCCACTGT CCCGGTCCTC TTCCCCTGAG

      2050       2060       2070       2080       2090       2100
GTCCCCTGAT ATCCCCTGGC TCAGTCCTAA CAAGACTGAG TCTTAACAAG ATGAGAAGTC

      2110       2120       2130       2140       2150       2160
CTCCCCTTCT TGCCTCCCAC AGTTTTCTTT GATGGGAGGT TTCAATAAAC AGCGATAAGA

      2170       2180       2190       2200       2210       2220
ACTCTAAAAA AAAAAAAAA. .......... .......... .......... ..........
```

Table 3

Base sequence of hOAT3 amino acid

```
                133                 142                 151                 160                 169                 178
5'  ATG ACC TTC TCG GAG ATC CTG GAC CGT GTG GGA AGC ATG GGC CAT TTC CAG TTC
    Met Thr Phe Ser Glu Ile Leu Asp Arg Val Gly Ser Met Gly His Phe Gln Phe

                187                 196                 205                 214                 223                 232
    CTG CAT GTA GCC ATA CTG GGC CTC CCG ATC CTC AAC ATG GCC AAC CAC AAC CTG
    Leu His Val Ala Ile Leu Gly Leu Pro Ile Leu Asn Met Ala Asn His Asn Leu

                241                 250                 259                 268                 277                 286
    CTG CAG ATC TTC ACA GCC GCC ACC CCT GTC CAC CAC TGT CGC CCG CCC CAC AAT
    Leu Gln Ile Phe Thr Ala Ala Thr Pro Val His His Cys Arg Pro Pro His Asn

                295                 304                 313                 322                 331                 340
    GCC TCC ACA GGG CCT TGG GTG CTC CCC ATG GGC CCA AAT GGG AAG CCT GAG AGG
    Ala Ser Thr Gly Pro Trp Val Leu Pro Met Gly Pro Asn Gly Lys Pro Glu Arg

                349                 358                 367                 376                 385                 394
    TGC CTC CGT TTT GTA CAT CCG CCG AAT GCC AGC CTG CCC AAT GAC ACC CAG AGG
    Cys Leu Arg Phe Val His Pro Pro Asn Ala Ser Leu Pro Asn Asp Thr Gln Arg

                403                 412                 421                 430                 439                 448
    GCC ATG GAG CCA TGC CTG GAT GGC TGG GTC TAC AAC AGC ACC AAG GAC TCC ATT
    Ala Met Glu Pro Cys Leu Asp Gly Trp Val Tyr Asn Ser Thr Lys Asp Ser Ile

                457                 466                 475                 484                 493                 502
    GTG ACA GAG TGG GAC TTG GTG TGC AAC TCC AAC AAA CTG AAG GAG ATG GCC CAG
    Val Thr Glu Trp Asp Leu Val Cys Asn Ser Asn Lys Leu Lys Glu Met Ala Gln

                511                 520                 529                 538                 547                 556
    TCT ATC TTC ATG GCA GGT ATA CTG ATT GGA GGG CTC GTG CTT GGA GAC CTG TCT
    Ser Ile Phe Met Ala Gly Ile Leu Ile Gly Gly Leu Val Leu Gly Asp Leu Ser

                565                 574                 583                 592                 601                 610
    GAC AGG TTT GGC CGC AGG CCC ATC CTG ACC TGC AGC TAC CTG CTG CTG GCA GCC
    Asp Arg Phe Gly Arg Arg Pro Ile Leu Thr Cys Ser Tyr Leu Leu Leu Ala Ala

                619                 628                 637                 646                 655                 664
    AGC GGC TCC GGT GCA GCC TTC AGC CCC ACC TTC GCC ATC TAC ATG GTC TTC CGC
    Ser Gly Ser Gly Ala Ala Phe Ser Pro Thr Phe Pro Ile Tyr Met Val Phe Arg

                673                 682                 691                 700                 709                 718
    TTC CTG TGT GGC TTT GGC ATC TCA GGC ATT ACC CTG AGC ACG GTC ATC TTG AAT
    Phe Leu Cys Gly Phe Gly Ile Ser Gly Ile Thr Leu Ser Thr Val Ile Leu Asn
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          727        736        745        754        763        772
GTG GAA TGG GTG CCT ACC CGG ATG CGG GCC ATC ATG TCG ACA GCA CTC GGG TAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Val Glu Trp Val Pro Thr Arg Met Arg Ala Ile Met Ser Thr Ala Leu Gly Tyr

          781        790        799        808        817        826
TGC TAC ACC TTT GGC CAG TTC ATT CTG CCC GGC CTG GCC TAC GCC ATC CCC CAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Cys Tyr Thr Phe Gly Gln Phe Ile Leu Pro Gly Leu Ala Tyr Ala Ile Pro Gln

          835        844        853        862        871        880
TGG CGT TGG CTG CAG TTA ACT GTG TCC ATT CCC TTC TTC GTC TTC TTC CTA TCA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Trp Arg Trp Leu Gln Leu Thr Val Ser Ile Pro Phe Phe Val Phe Phe Leu Ser

          889        898        907        916        925        934
TCC TGG TGG ACA CCA GAG TCC ATA CGC TGG TTG GTC TTG TCT GGA AAG TCC TCG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ser Trp Trp Thr Pro Glu Ser Ile Arg Trp Leu Val Leu Ser Gly Lys Ser Ser

          943        952        961        970        979        988
GAG GCC CTG AAG ATA CTC CGG CGG GTG GCT GTC TTC AAT GGC AAG AAG GAA GAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Glu Ala Leu Lys Ile Leu Arg Arg Val Ala Val Phe Asn Gly Lys Lys Glu Glu

          997       1006       1015       1024       1033       1042
GGA GAA AGG CTC AGC TTG GAG GAG CTC AAA CTC AAC CTG CAG AAG GAG ATC TCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Gly Glu Arg Leu Ser Leu Glu Glu Leu Lys Leu Asn Leu Gln Lys Glu Ile Ser

         1051       1060       1069       1078       1087       1096
TTG GCC AAG GCC AAG TAC ACC GCA AGT GAC CTG TTC CGG ATA CCC ATG CTG CGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Leu Ala Lys Ala Lys Tyr Thr Ala Ser Asp Leu Phe Arg Ile Pro Met Leu Arg

         1105       1114       1123       1132       1141       1150
CGC ATG ACC TTC TGT CTT TCC CTG GCC TGG TTT GCT ACC GGT TTT GCC TAC TAT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Arg Met Thr Phe Cys Leu Ser Leu Ala Trp Phe Ala Thr Gly Phe Ala Tyr Tyr

         1159       1168       1177       1186       1195       1204
AGT TTG GCT ATG GGT GTG GAA GAA TTT GGA GTC AAC CTC TAC ATC CTC CAG ATC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ser Leu Ala Met Gly Val Glu Glu Phe Gly Val Asn Leu Tyr Ile Leu Gln Ile

         1213       1222       1231       1240       1249       1258
ATC TTT GGT GGG GTC GAT GTC CCA GCG AAG TTC ATC ACC ATC CTC TCC TTA AGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ile Phe Gly Gly Val Asp Val Pro Ala Lys Phe Ile Thr Ile Leu Ser Leu Ser

         1267       1276       1285       1294       1303       1312
TAC CTG GGC CGG CAT ACC ACT CAG GCC GCT GCC CTG CTC CTG GCA GGA GGG GCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Tyr Leu Gly Arg His Thr Thr Gln Ala Ala Ala Leu Leu Leu Ala Gly Gly Ala
```

```
      1321        1330        1339        1348        1357        1366
ATC TTG GCT CTC ACC TTT GTG CCC TTG GAC TTG CAG ACC GTG AGG ACA GTA TTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ile Leu Ala Leu Thr Phe Val Pro Leu Asp Leu Gln Thr Val Arg Thr Val Leu

      1375        1384        1393        1402        1411        1420
GCT GTG TTT GGG AAG GGA TGC CTA TCC AGC TCC TTC AGC TGC CTC TTC CTG TAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ala Val Phe Gly Lys Gly Cys Leu Ser Ser Ser Phe Ser Cys Leu Phe Leu Tyr

      1429        1438        1447        1456        1465        1474
ACA AGT GAA TTA TAC CCC ACA GTC ATC AGG CAA ACA GGT ATG GGC GTA AGT AAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Thr Ser Glu Leu Tyr Pro Thr Val Ile Arg Gln Thr Gly Met Gly Val Ser Asn

      1483        1492        1501        1510        1519        1528
CTG TGG ACC CGC GTG GGA AGC ATG GTG TCC CCG CTG GTG AAA ATC ACG GGT GAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Leu Trp Thr Arg Val Gly Ser Met Val Ser Pro Leu Val Lys Ile Thr Gly Glu

      1537        1546        1555        1564        1573        1582
GTA CAG CCC TTC ATC CCC AAT ATC ATC TAC GGG ATC ACC GCC CTC CTC GGG GGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Val Gln Pro Phe Ile Pro Asn Ile Ile Tyr Gly Ile Thr Ala Leu Leu Gly Gly

      1591        1600        1609        1618        1627        1636
AGT GCT GCC CTC TTC CTG CCT GAG ACC CTG AAT CAG CCC TTG CCA GAG ACT ATC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ser Ala Ala Leu Phe Leu Pro Glu Thr Leu Asn Gln Pro Leu Pro Glu Thr Ile

      1645        1654        1663        1672        1681        1690
GAA GAC CTG GAA AAC TGG TCC CTG CGG GCA AAG AAG CCA AAG CAG GAG CCA GAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Glu Asp Leu Glu Asn Trp Ser Leu Arg Ala Lys Lys Pro Lys Gln Glu Pro Glu

      1699        1708        1717        1726        1735        1744
GTG GAA AAG GCG TCC CAG AGG ATC CCT CTA CAG CCT CAC GGA CCA GGC CTG GGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Val Glu Lys Ala Ser Gln Arg Ile Pro Leu Gln Pro His Gly Pro Gly Leu Gly

      1753
TCC AGC TGA 3'
--- --- ---
Ser Ser ***
```

Example 6

Identification of hOAT3 function

By using T7 RNA polymerase, cRNA (RNA complementary to cDNA) was prepared in vitro from the plasmid comprising the hOAT3 recovered above by the method by Sekine, et al. (see Sekine, T., et al. J. Biol. Chem., Vol. 272, pp. 18526-9, 1997).

According to the already reported method of Sekine, et al. (Sekine, T., et al. J. Biol. Chem., Vol. 272, pp. 18526-9, 1997), the resulting hOAT3 cRNA was injected in the Xenopus oocyte; the oocyte was subjected to an uptake test with various radiolabeled organic anions and organic cations. The control oocyte cell (oocyte cell with no injection of hOAT3 cRNA) and the oocyte cell injected with hOAT3 cRNA were cultured in a buffer containing the following radiolabels for one hour, to assay the uptake of the radiolabels into the oocytes.

The results are shown in Figs. 6 to 18. In each figure, open column expresses the case of the control oocyte used; and closed column expresses the case of the oocyte injected with hOAT3 cRNA. Fig. 6 depicts the uptake activity of $^{14}$C-PAH (p-aminohippuric acid) (10 μM); Fig. 7 depicts the uptake activity of $^{3}$H-estrone sulfate (50 nM); Fig. 8 depicts the uptake activity of $^{3}$H-dehydroepiandrosterone sulfate (50 nM); Fig. 9 depicts the uptake activity of $^{3}$H-ochratoxin A (100 nM); Fig. 10 depicts the uptake activity of $^{3}$H-cimetidine (150 nM); Fig. 11 depicts the uptake activity of $^{3}$H-estradiol glucuronide (50 nM); Fig. 12 depicts the uptake activity of $^{3}$H-prostaglandin E2 (1 nM); Fig. 13 depicts the uptake activity of $^{14}$C-taurocholic acid (10 μM); Fig. 14 depicts the uptake activity of $^{14}$C-glutaric acid (10 μM); Fig. 15 depicts the uptake activity of $^{3}$H-methotrexate (100 nM); Fig. 16 depicts the uptake activity of $^{14}$C-salicylic acid (1 μM); Fig. 17 depicts the uptake activity of $^{14}$C-

indomethacin (10 μM); and Fig. 18 depicts the uptake activity of $^{14}$C-cholic acid (10 μM).

As shown in these figures, the values of these radiolabels in the oocyte with hOAT3 expression were higher than the values thereof in the control oocyte, suggesting that hOAT3 transported these compounds.

Consequently, the oocyte with hOAT3 expression takes up $^{14}$C-PAH (p-aminohippuric acid), $^{3}$H-estrone sulfate, $^{3}$H-dehydroepiandrosterone sulfate, $^{3}$H-ochratoxin A, $^{3}$H-cimetidine, $^{3}$H-estradiol glucuronide, $^{3}$H-prostaglandin E2, $^{14}$C-taurocholic acid, $^{14}$C-glutaric acid, $^{3}$H-methotrexate, $^{14}$C-salicylic acid, $^{14}$C-indomethacin, and $^{14}$C-cholic acid. On contrast, hOAT3 never transported the typical organic cation $^{14}$C-TEA (tetraethylammonium) (not shown in the figures).

Then, the hOAT3 transport of organic anions was examined at the Michaelis-Menten kinetic test. By examining the change in the hOAT3 uptake of estrone sulfate and methotrexate at various concentrations, the dependency of the OAT3 transport on the concentrations of these substances was examined. The uptake experiment of radiolabeled estrone sulfate and methotrexate was carried out by using the oocyte injected with hOAT3 cRNA and the control oocyte (with no injection of cRNA), by the method described above. Consequently, the Km values of estrone sulfate and methotrexate were 3.08 μM and 2.22 μM, respectively.

So as to examine the substrate selectivity of hOAT3, various anionic substances were added to the $^{3}$H-estrone sulfate uptake experiment system with the oocyte injected with hOAT3 cRNA, to examine their influences (inhibition experiment).

The $^{3}$H-estrone sulfate uptake experiment was conducted by using the oocyte injected with hOAT3 cRNA according to the method described above.

More specifically, the control oocyte (oocyte with no injection of hOAT3 cRNA) and the oocyte with injection of hOAT3 cRNA were cultured in a buffer containing 50 nM $^{3}$H-estrone sulfate alone or containing non-radiolabeled compounds at 500 μM or the concentration shown in the figure for one hour, to assay the uptake of $^{3}$H-estrone sulfate. When the uptake of 50 nM $^{3}$H-estrone sulfate singly contained in the buffer into the oocyte with injection of hOAT3 cRNA was designated 100 %, the individual uptake values in the buffer containing inhibitory agents were expressed in %.

The results are shown in Fig. 19. As shown in Fig. 19, all these compounds inhibited the uptake of $^{3}$H-estrone sulfate into the oocyte injected with hOAT3 cRNA, indicating that these compounds were interactive with hOAT3. Consequently, it was indicated that various anionic substances (estrone sulfate, PAH, taurocholic acid, probenecid, furosemide, zidovudine, penicillin G, BSP, glutaric acid, indomethacin, and methotrexate) significantly inhibited the transport of $^{3}$H-estrone sulfate with hOAT3 (see Fig. 19). Alternatively, tetraethylammonium as one of typical organic cations never exerted any inhibitory action. Based on these results, it is evidenced that the inventive hOAT3 is a multi-selective organic anion transporter.

Industrial Applicability

The invention provides a novel organic anion transporter with wide substrate selectivity of organic anions and in selective distribution in brain and liver and the like.

The inventive organic anion transporter is involved in the uptake of various drugs in cells and is also involved in the dynamics of drugs in biological organisms. Therefore, the inventive organic anion transporter is useful not only for the cell

viability and activation but also for the screening of pharmacokinetics.